INVESTIGATIONS OF SOME INFLAMMATORY AND OTHER BIOMARKERS DURING MALARIA INFECTIONS IN CHILDREN

By

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A thesis submitted in partial fulfillment of the requirement for the degree of M.Sc. in Biochemistry Faculty of Veterinary Medicine, University of Khartoum

October 2008

DEDICATION

To:

The soul of my father with great love

My mother whose continuously lively dedication, and Support remained an unlimited source of encouragement for me.

My brothers and sisters who provided a healthy and conductive environment around me allowing this effort to materialize.

ACKNOWLEDGEMENTS

I pray to Allah thankfully whose will permitted the completion of this work.

I am greatly indebted to my supervisor Prof. Asia Mohammed El Hassan for her valuable suggestions, advice, guidance, continuous encouragement and patience throughout my study.

I am especially grateful to Prof. Onn Hajji Hashim the Head of the Department of Molecular Medicine, Faculty of Medicine, University of Malaya, Malaysia for his generous assistance by allowing me to do the analysis of the samples in their University laboratories.

Thanks are extended to Mr. Mohammed Hamato and Mr. Mustafa Zubbiedi for their kind help during the laboratory investigations.

Sincere thanks are extended to my teachers, friends and my family for their support and encouragement.

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ABSTRACT

Introduction and literature review:

Malaria is a distressing health problem that posses a socio-economic burden of considerable magnitude for communities in developing countries. It causes some immunological and hematological changes particularly inflammatory biomarkers and hemoglobin. Research is extensively required in this area to promote health services and to target radical solution to the problem.

The objective of the study is to investigate some inflammatory and other biomarkers during malaria infections in some of Sudanese children in Sinar State and how these biomarkers may relate to factors such as age and hemoglobin which in children may be important in disease progression and control.

Materials and Methods:

Thirty-two samples of blood were collected from 16 malaria patients from Sinar hospital and 16 apparently healthy individuals from the same area as control group from which serum was separated.

The investigations were carried out to estimate serum C-reactive protein (CRP), tumor necrosis factor alpha (TNF α) and serum magnesium in the two groups.

High sensitive and highly specific techniques were used for biomarker determinations.

Results:

Results showed that children of different age groups with Malaria have significantly higher levels of C-reactive protein.

Compared with the control, serum magnesium levels were below normal ranges in the malaria patients, while serum tumor necrosis factor was significantly high among malaria patients. The hemoglobin level was low in most of infected children, there was no correlation between serum magnesium levels and hemoglobin.

Discussion and Conclusion:

It is concluded that malaria is one of the causes of biomarker changes. C-reactive protein concentrations were increased in malaria patients and of high incidence among children. TNF level was increased in patients .Serum magnesium level was decreased in malaria patients in this study, The increase of CRP and TNF concentration may be attributed to the severe inflammation in children as they known as inflammatory bio markers . but the hemoglobin which was low in the infected children was due to anemia and bad nutrition status. There was negative correlation between serum magnesium and hemoglobin levels.

ملخص البحث

المقدمة ومراجعة الادب:

تعتبر الملاريا مشكلة صحية تؤرق المجتمعات بدرجة كبيـرة بتأثيرهـا اجتماعيا واقتصاديا وتسبب تغيرات دموية ومناعية خصوصاً المؤشرات الحيويـة الالتهابية وخضاب الدم والابحاث فى هذا المجال ضرورية ومطلوبـة لتحـسين الخدمات الصحية استهدافاً لحل شامل للمشكلة.

الهدف من الدراسة هو فحص واختبار بعض هذه المؤشـرات الالتهابيـة والمؤشرات الأخرى معملياً أثناء الإصابة بالملاريا عند بعض الأطفال السودانيين بولاية سنار ودراسة ارتباط هذه المؤشرات بالعوامل الاخـري كـالعمر ونـسبة خضاب الدم والذي يمكن أن يكون مهماً فى تطور المرض أو التحكم فيه.

المواد وطرق البحث:

أخذت اثنتان وثلاثون عينة من الدم 16 مصابين بالملاريا مـن مستـشفى سنار و16 آخرين أصحاء من نفس المنطقة يستخدمون كمجموعة مراقبة حيث تم فصل الدم منها.

أجريت الاختبارات المعملية لتقدير كميات CRP وعامـل نخـر الـورم وعنصر الماغنيزيوم في مصل الدم وذلك للمجموعتين باسـتخدام طـرق دقيقـة وحساسة لقياس المؤشرات الحيوية الالتهابية.

النتائج:

أوضحت النتائج أن الاطفال المصابين بالملاريا بمختلف فئاتهم العمرية قد زادت مستويات CRP معنويا عندهم .

مقارنة بالأصحاء، مستوي الماغنيزيوم في مصل الدم وجد انه تحت المدى الطبيعي عند الأطفال المصابين بالملاريا بينما كان عامل نخر الـورم مرتفـع معنويـاً وسـط المـصابين بالملاريـا أمـا الهيموغلـوبين فقـد كـان منخفضا عند معظم الأطفال المصابين ولا توجد علاقة بينه وبين خضاب الدم.

المناقشة والخاتمة:

خلصنا إلي أن الملاريا هي احد أسباب تغيرات المؤشرات الالتهابيـة ، CRP زادت تراكيزه عند مرضى الملاريا وبدرجات كبيرة عند الأطفال كمـا أن عامل نخر الدم زادت مستوياته عند المرضى أما الماغنيزيوم في مصل الدم فقـد كان منخفضا فى هذه الدراسة وايضاً لا توجد علاقة واضحة بين الماغنيزيوم في المصل ومستوى خضاب الدم . زيادة تراكيزCRP و TNF ربما تعزى للحالة الالتهابية الشديدة عند الأطفال التي عرفت كمؤشرات التهابية أما الهيموغلوبين فقد نقص مستواه في الدم عند معظم الأطفال المصابين وذلك نتيجة للأنيميا المصاحبة والحالة الغذائية السيئة .كما توجد علاقة سلبية بين مستويات الماغنيزيوم وخضاب الدم .

INTRODUCTION

Malaria is a serious infectious disease spread by mosquitoes and endemic in many developing countries. It is common in tropical climates and is characterized by chills, fevers, and an enlarged spleen. Malaria is caused by protozoan parasites of the genus Plasmodium. In humans malaria is caused by *P. falciparum*, *P. malarae*, *P. ovale*, and *P. vivax*. However, *P. falciparum* is the most important cause of the disease and responsible for about 80% of malaria infections (Mendis *et al*., 2000).

Malaria causes about 350–500 million infections in humans and approximately one to three million deaths annually (Breman, 2001). The vast majority of cases occur in children under the age of 5 years (Greenwood *et al.*, 2005). Pregnant women are especially vulnerable. Despite efforts since 1992 to reduce transmission and increase treatment, there has been little change in areas at risk of this disease (Hay. *et al.*, 2004). Methods used to prevent the spread of the disease, or to protect individuals in areas where malaria is endemic, include drugs, mosquito eradication, and the prevention of mosquito bites. Currently there is no vaccine that will prevent malaria, but this is an active field of research. It is hoped that the sequencing of the *P. falciparum* genome will provide targets for new drugs or vaccines (Gardner *et al*., 2002).

Over the past few decades, a literature has emerged that argues for most of the pathology seen in all of these infectious diseases being explained by activation of the inflammatory system, with the balance

between the pro- and anti-inflammatory cytokines being tipped towards the onset of systemic inflammation.

There is now remarkably widespread acceptance that cytokines such as TNF and interleukin-1 constitute the essential mechanism of systemic disease caused by infectious agents. Indeed, one would be pressed to find an alternative explanation for the anorexia, tiredness, aching joints and muscles, fever and sleepiness that patients experience in any systemic infection, including both vivax and falciparum malaria (Ubalee *et al*., 2001). Tumor necrosis factor alpha (TNF-α) is thought to play a role in the development of immunity and pathology in malaria infections in experimental models and in humans (Clark *et al.,* 1992). In fact, the genetic susceptibility to severe forms of falciparum malaria is differentially associated with TNF-α promoter gene polymorphisms (Ubalee *et al*., 2001). As such TNF can be considered as a biomarker for malaria.

A biomarker is a characteristic that can be objectively measured and evaluated as an indicator of normal biological or pathogenic processes as well as pharmacological responses to a therapeutic intervention. Interest in biomarkers application for diagnostics and drug discovery as well as drug development has increased remarkably since the beginning of the 21st century. Biomarkers are useful not only for diagnosis of some of diseases but also for understanding the pathogenic mechanisms involved in disease (Clark *et al.,* 1992)..

The information generated by testing for biomarkers can be used for three purposes:

1. The assessment of needs and the planning of interventions to improve health.
2. The monitoring of changes in health and the evaluation of interventions.
3. Lobbying for changes in policies to address population health needs.

Currently, no information is available about the biochemical or molecular signatures of severe and complicated malaria or mild and asymptomatic malaria. The detection of biomarkers of severe malaria, along with traditional microscopy, could result in effective management of malaria particularly in more vulnerable groups such as children.

Other biomarkers of inflammatory response, if malaria is considered as one; may include inflammatory interleukins such as IL-1, IL-6, IL-8 and IL-18 and acute phase proteins such as C-reactive protein. A distinct molecular variant of disease-specific CRP was affinity purified from sera of malaria patients (CRP_{Mal}). This CRP showed strong binding with malaria erythrocytes (RBC_{Mal}) (Joy *et al.,* 2003). These studies provide direct evidence for an important functional interaction of this acute-phase protein by triggering the CRP-complement pathway after it binds with RBC_{Mal}. Hemolysis as triggered by this pathway may be one of the causative factors of anemia, a common clinical manifestation of malaria to which over half of malaria-related deaths are attributed (Murphy *et al.*, 2006).

Since red blood cells contain high amounts of magnesium, hemolysis might result in increased serum magnesium. This offers a potential application of this element as a biomarker of acute falciparum malaria infection in adults (Rudin *et al*., 1997).

Objectives:

General objective:

- To study the level of some inflammatory and other biomarkers during malarial infections.

Specific objective:

- To examine the serum levels of c-reactive proteins, tumor necrosis factor alpha in malarial infection.
- To measure serum magnesium and hemoglobin level during malarial infections.
- To investigate the correlation of magnesium with hemoglobin levels.

CHAPTER ONE
LITERATURE REVIEW

1.1 Malaria infection:

Malaria has infected humans for over 50,000 years, and may have been a human pathogen for the entire history of our species (Joy *et al.,* 2003). The term malaria originates from Medieval Italian: *mala aria* - "bad air"; and the disease was formerly called *ague* or *marsh fever* due to its association with swamps.

Although the blood stage and mosquito stages of the malaria life cycle were established in the 19th and early 20th centuries, it was not until the 1980s that the latent liver form of the parasite was observed (Krotoski *et al.,* 1982). The discovery of this latent form of the parasite finally explained why people could appear to be cured of malaria but still relapse after the parasite had disappeared from their blood streams.

Infection with *Plasmodium falciparum* can also lead to severe malaria, a heterogeneous syndrome involving respiratory and neurological disturbances with significant mortality rate (Marsh. *et al.,* 1996).

1.1.1 Malaria epidimiolgy:

Indeed, if the prevalence of malaria stays on its present upwards course, the death rate could double in the next twenty years (Breman, 2001). Precise statistics are unknown because many cases occur in rural areas where people do not have access to hospitals or the means to afford health care. Consequently, the majority of cases are undocumented (Breman, 2001).

The geographic distribution of malaria within large regions is complex, malarial and malaria-free areas are often found close to each other (Hay *et al.,* 2004). In drier areas, outbreaks of malaria can be predicted with reasonable accuracy by mapping rainfall (Grover-Kopec *et al.,* 1998). Malaria is presently endemic in a broad band around the equator, in areas of the Americas, many parts of Asia, and much of Africa as indicated in figure (1).

However, it is in sub-Saharan Africa where 85– 90% of malaria fatalities occur (Scott and Layne, 2000). Malaria is more common in rural areas than in cities (Hay *et al.,* 2004). For example, the cities of Vietnam, Laos and Cambodia are essentially malaria-free, but the disease is present in many rural regions in South East Asia (Trung *et al.,* 2004). By contrast, in Africa malaria is present in both rural and urban areas, though the risk is lower in the larger cities due to higher levels of education and awareness of the problem and also because of the provision of better health services (Joy *et al.,* 2003).

1.1.2 Socio-economic effects of malaria:

Malaria is not just a disease commonly associated with poverty, but is also a cause of poverty and a major hindrance to economic development. The disease has been associated with major negative economic effects on regions where it is widespread. In its entirety, the economic impact of malaria has been estimated to cost Africa 12 billion US$ every year (Breman, 2001). The economic impact includes costs of of health care, working days lost due to sickness, days lost in education,

decreased productivity due to brain damage from cerebral malaria, and loss of investment and tourism. (Greenwood and Mutabingwa, 2002).

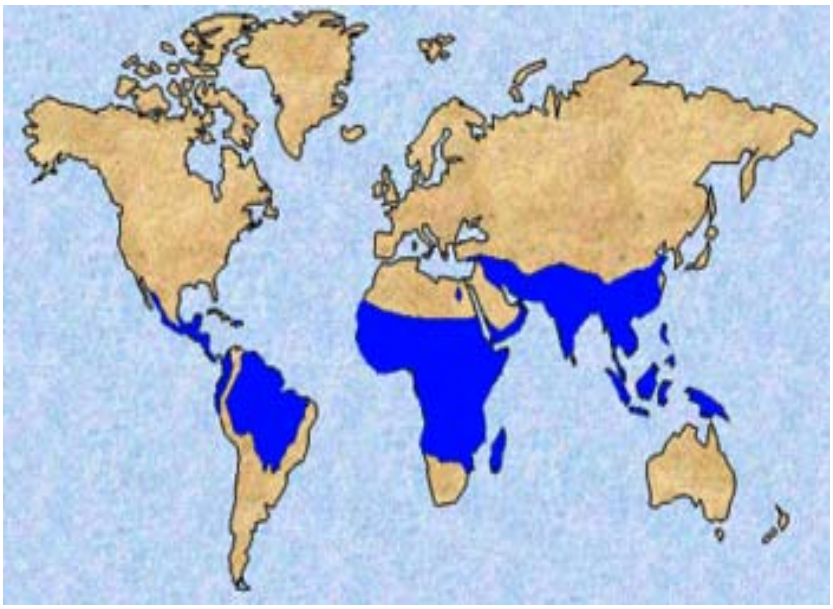

Fig. (1): Areas of the world where malaria is endemic (coloured blue)
Source: (Hay *et al.*, 2004).

In some countries with a heavy malaria burden, the disease may account for as much as 40% of public health expenditure, 30-50% of inpatient admissions, and up to 50% of outpatient visits that it acounts as a real devastation for the economy and prosperity of developing countries (Joy *et al.*, 2003).

1.1.3 Symptoms of malaria:

Symptoms of malaria vary a great deal and may include fever, shivering, joint pain, vomiting, anemia caused by hemolysis, hemoglobinuria, and convulsions. There may be the feeling of tingling in the skin, particularly with malaria caused by *P. falciparum*. The classical symptom of malaria is cyclical occurrence of sudden coldness followed by rigor and then fever and sweating lasting four to six hours, occurring every two days in *P. falciparum*, *P. vivax, P. ovale* infections, and every three days in *P. malariae* (Marsh *et al.,* 1996).For reasons that are poorly understood, but which may be related to high intracranial pressure, children with malaria frequently exhibit abnormal posturing, a sign that indicates severe brain damage (Idro *et al.,* 2007).

1.1.4 Consequences of malaria:

Malaria has been found to cause cognitive impairments, especially in children (Boivin, 2002). The widespread anemia that malaria precipitates during a period of rapid brain development may result in brain damage. This neurologic damage may also result from cerebral malaria to which children are more vulnerable (Boivin, 2002).

Malaria which almost exclusively caused by *P. falciparum* is a severe infection and usually arises 6-14 days after infection (Kain *et al.,* 1998). Consequencies of severe malaria include coma and death if untreated particularly in vulnerable individuals. Splenomegaly, severe headache, cerebral ischemia, hepatomegaly, hypoglycemia, and hemoglobinuria with renal failure may occur. Renal failure may cause blackwater fever, where hemoglobin from lysed red blood cells leaks into

the urine. Severe malaria can progress extremely rapidly and cause death within hours or days (Kain *et al.,* 1998). In the most severe cases of the disease fatality rates can exceed 20%, even with intensive care and treatment (Kain *et al.,* 1998). In endemic areas, treatment is often less satisfactory and the overall fatality rate for all cases of malaria can be as high as one in ten (Mockenhaupt *et al*., 2004). Over the longer term, developmental impairments have been documented in children who have suffered episodes of severe malaria (Carter *et al.,* 2005). Chronic malaria is that seen in both *P. vivax* and *P. ovale*, but not in *P. falciparum*. Here, the disease can relapse months or years after exposure, due to the presence of latent parasites in the liver. The longest incubation period reported for a *P. vivax* infection is 30 years (Carter *et al.,* 2005).

1.1.5 Mosquito vectors and the *Plasmodium* life cycle:

The parasite's primary (definitive) hosts and transmission vectors are female mosquitoes of the *Anopheles* genus. (Two females of anopheles mosquito full of blood shown in Fig .3). Young mosquitoes first ingest the malaria parasite by feeding on an infected human carrier and the infected *Anopheles* mosquitoes carry *Plasmodium* sporozoites in their salivary glands. Once ingested, the parasite gametocytes taken up in the blood will further differentiate into male or female gametes and then fuse in the mosquito gut. This produces an ookinete that penetrates the gut lining and produces an oocyst in the gut wall. When the oocyst ruptures, it releases sporozoites that migrate through the mosquito's body to the salivary glands, (the Plasmodium life cycleis shown in Fig-2), where they are then ready to infect a new human host. This type of

transmission is occasionally referred to as anterior station transfer (Kain *et al.,* 1998).

Only female mosquitoes feed on blood, thus males do not transmit the disease. The females of the *Anopheles* genus prefer to feed at night. They usually start searching for a meal at dusk, and will continue throughout the night. Malaria parasites can also be transmitted by blood transfusions, although this is rare. A longitudinal study was conducted between October 1989 and February 1990 in a malaria holoendemic area of Gabon to determine the plasma concentration of various cytokines in individuals continuously exposed to infection with malaria parasites. No cases of severe malaria were seen and fever was the main presenting symptom of clinical malaria. Parasite rates were highest in children 6-9 years old but clinical malaria was seen essentially in children below 6 years of age (Carter *et al.,* 2005).

1.1.6 The Malaria parasites:

Malaria is caused by protozoan parasites of the genus *Plasmodium* (phylum Apicomplexa). Parasitic *Plasmodium* species also infect birds, reptiles, monkeys, chimpanzees and rodent (Escalante and Ayala, 1994). There have been documented human infections with several simian species of malaria (Mens *et al.,* 2006).

One aspect of severe malaria pathogenesis is an excessive or dysregulated inflammatory response to infection. With the characterization of Toll-like receptors (TLRs), which initiate inflammation upon detection of microbial products, involvement of TLRs in the host response to malaria has undergone intense investigation.

While TLRs appear to mediate inflammation in malaria infection and may contribute to development of severe malaria, it is unlikely that they operate in isolation from other components of innate immunity (Beare 2006).

1.1.7 Diagnosis

The most economic, preferred, and reliable diagnosis of malaria is microscopic examination of blood films because each of the four major parasite species has distinguishing characteristics. Two sorts of blood

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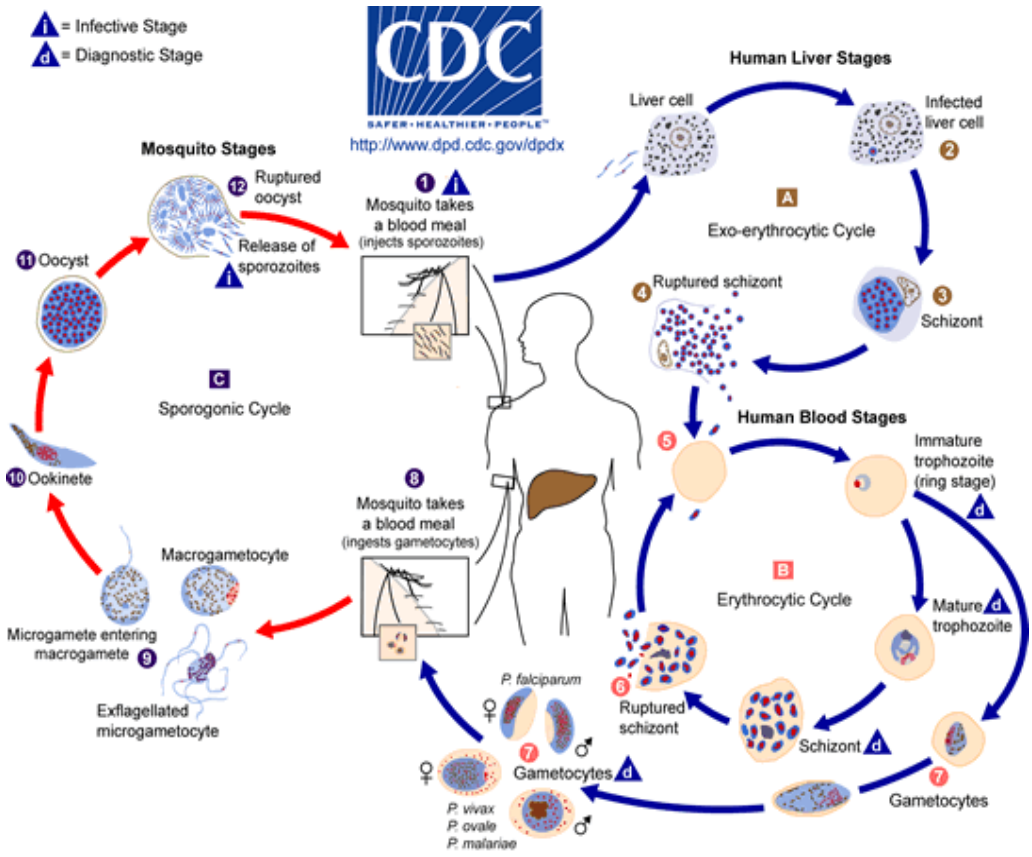


Fig. (2). Plasmodium life cycle (Source: Beare, 2006).

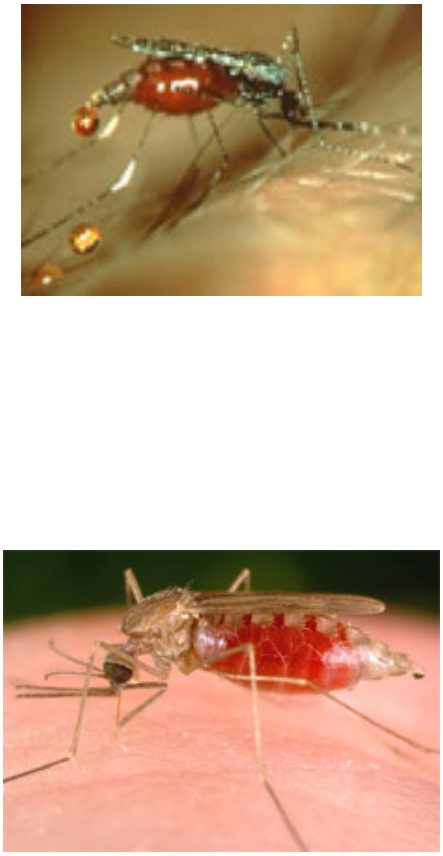


Figure (3): Two females of anopheles mosquito full of blood (Beare, 2006).

films are traditionally used. Thin films allow species identification because the parasite's appearance is best preserved in this preparation. Thick films allow the laboratory technologist to screen a larger volume of blood and are about eleven times more sensitive than the thin film. Thus picking up low levels of infection is easier on the thick film, but the appearance of the parasite is much more distorted and therefore distinguishing between the different species can be much more difficult. It is imperative to utilize both smears while attempting to make a definitive diagnosis (Mens *et al.,* 2006).

Molecular methods such as rapid real-time assays based on the polymerase chain reaction are available in some clinical laboratories (Mens *et al.,* 2006). They are being developed with the hope to deploy them in endemic areas.

Severe malaria is commonly misdiagnosed in Africa. In malaria-endemic areas, parasitemia does not ensure a diagnosis of severe malaria because parasitemia can be incidental to other concurrent disease. Recent investigations suggest that malarial retinopathy is better than any other clinical or laboratory feature in distinguishing malarial from non-malarial coma (Beare, 2006).

1.1.8 Malaria prognosis:

The parasite is relatively protected from attack by the body's immune system because for most of its human life cycle it resides within the liver and blood cells and is relatively invisible to immune surveillance. However, circulating infected blood cells are destroyed in the spleen as a part of innate immune response to malaria . To avoid this fate, the *P. falciparum* parasite displays adhesive proteins on the surface

of the infected blood cells, causing the blood cells to stick to the walls of small blood vessels, thereby sequestering the parasite from passage through the general circulation and the spleen (Chen *et al.*, 2000). This "stickiness" is the main factor giving rise to hemorrhagic complications of malaria. High endothelial venules (the smallest branches of the circulatory system) can be blocked by the attachment of masses of these infected red blood cells (Mens *et al.*, 2006).

1.1.9 Malaria and anemia:

Anemia commonly occurs in chronic infection, inflammation, and malignancy. This type of anemia is characterized by decreased plasma iron and iron-binding capacity, normal or elevated iron stores, modest decrease of red cell survival, and relative failure of bone marrow to increase red cell production and normal hemglobin level. It was first described with infection, because of its association with other types of chronic diseases such as chronic inflammation and cancer, it is called anemia of chronic diseases (ACD). An understanding of the role of inflammatory cytokines in the most of the diseases underlying ACD has suggested that such Inflammatory cytokines are important factors in the pathophysiology of ACD . TNF and IL-1 have effects on iron metabolism and red cell survival *in vivo,* inflammatory cytokines have also been implicated in the inappropriately low erythropoietin (EPO) levels in ACD patients (Galasko D. 2001).

Nutrition plays a major role in maintaining health, and malnutrition appears to generate vulnerability to a wide variety of diseases and general ill health. Opinions are mixed regarding how under

nutrition, whether it is characterized in terms of growth faltering or micronutrient malnutrition, affects susceptibility to malarial illness and mortality (Chen *et al.,* 2000).

1.1.10 Treatment:

Treatment of malaria involves supportive measures as well as specific antimalarial drugs. When properly treated, someone with malaria can expect a complete cure (Boivin, 2002).

1.1.10.1 Antimalarial drugs:

Most drugs used in the treatment of malaria are active against the parasitic form in the blood (the form that causes the disease) and include:

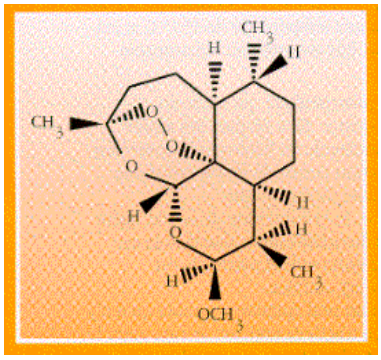
- chloroquine
- sulfadoxine-pyrimethamine (Fansidar®)
- mefloquine (Lariam®)
- atovaquone-proguanil (Malarone®)
- quinine
- Doxycycline
- artemisin derivatives

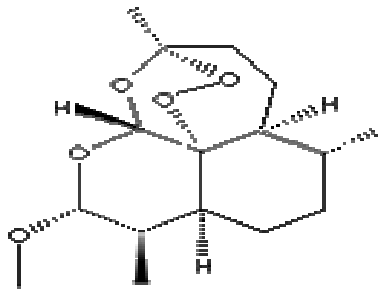
In addition, primaquine is active against the dormant parasitic liver forms (hypnozoites) and prevents relapses.

Chloroquine is very cheap and until recently, was very effective, which made it the antimalarial drug of choice for many years in most parts of the world. However, resistance of *Plasmodium falciparum* to chloroquine has spread recently from Asia to Africa, making the drug ineffective against the most dangerous Plasmodium strain in many

affected regions of the world. In those areas where chloroquine is still effective it remains the first choice. Unfortunately, chloroquine-resistance is associated with reduced sensitivity to other drugs such as quinine and amodiaquine (Killeen *et al*., 2002).

Artemisinin is a new antimalarial drug of Chinese origin, derived from the herb Artemisia annua L., (sweet wormwood) belonging to the family of Asteraccae.





Artmethinin

Figure (4): Artmethinin chemical structure (Mens *et al.*, 2006).

1.1.10.2 Vaccination:

Asafe and effective vaccine would have been the easiest way to control malaria . however after decates of search avaccine is still ellusive. The first promising studies demonstrating the potential for a malaria vaccine were performed in 1967 by immunizing mice with live, radiation-attenuated sporozoites, providing protection to about 60% of the mice upon subsequent injection with normal, viable sporozoites (Matuschewski, 2006). Since the 1970s, there has been a considerable effort to develop similar vaccination strategies for humans.

The complex life cycle of the parasite involving human and vector mosquitoes as well as its allelic diversity and antigenic variations makes the development and implementation of effective malaria control intervention problematic. The immune response that correlates with protection in these lifelong residents of endemic areas has not been identified, so development of a vaccine that mimics this immunity will be difficult (Galasko, 2001).

It is now becoming evident that multi-intervention approach may be the most appropriate way of combating malaria in view of the increasing resistance of the parasite to antimalarial drugs as well as vector mosquitoes to insecticides. Recent developments, including better methods for antigen production, improved adjuvants and novel delivery systems, provide optimism that sustained and appropriate long-lived immunity can be achieved (Matuschewski, 2006).

Presently, there is a huge variety of vaccine candidates. Pre-erythrocytic vaccines are vaccines that target the parasite before it reaches the blood. Other vaccine candidates include: those that seek to induce immunity to the blood stages of the infection; those that seek to avoid more severe pathologies of malaria by preventing adherence of the parasite to blood venules and placenta; and transmission-blocking vaccines that would stop the development of the parasite in the mosquito right after the mosquito has taken a bloodmeal from an infected person. It is hoped that the sequencing of the *P. falciparum* genome will provide targets for new drugs or vaccines.(Killeen, *et al*. 2002).

1.1.10.3 Prevention and disease control:

Mosquito nets help to keep mosquitoes away from people, and thus greatly reduce the infection and transmission of malaria. The nets are not a perfect barrier, so they are often treated with an insecticide designed to kill the mosquito before it has time to search for a way past the net. Insecticide-treated nets (ITN) are estimated to be twice as effective as untreated nets (Hull and Kevin, 2006) and offer greater than 70% protection compared with no net (Bachou, *et al*., 2006), Since the Anopheles mosquitoes feed at night, the preferred method is to hang a large "bed net" above the center of a bed such that it drapes down and covers the bed completely.

Prevention is an important component of Malaria control in endemic countries is achieved through:

- Preventing infection, by avoiding bites by parasite-carrying mosquitoes.
- Preventing disease.

- Vector control
- Personal protection measures such as insecticide-treated bed nets
- Mosquito bite detergents, creams and sprays .
- Preventive treatment with antimalarial drugs of vulnerable groups such as pregnant women, who receive intermittent preventive treatment.

1.2 Biomarkers:

1.2.1 Definition of biomarkers:

Biological markers (biomarkers) have been defined by Hulka and colleagues (Hulka,1990) as "cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids. More recently, the definition has been broadened to include biological characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (Naylor, 2003).

Biomarkers of all types have been used by generations of epidemiologists, physicians, and scientists to study human disease. The application of biomarkers in the diagnosis and management of cardiovascular disease, infections, immunological and genetic disorders, and cancer are well known (Hulka, 1990). Their use in research has served more than a purpose (Gordis, 1996). Neuroscientists have relied on biomarkers such as β-amyloid protein in Alzheimer disease to assist in the diagnosis and treatment of nervous system disorders and to investigate their cause (Verbeek, *et al.,* 2003).

The rapid growth of molecular biology and laboratory technology has expanded to the point at which the application of technically advanced biomarkers will soon become even more feasible (Verbeek *et al.,* 2003).

1.2.2 Types of biomarkers:

Biomarkers have been classified by Perera and Weinstein (2000) on the basis of the sequence of events from exposure to disease manifestation. Schulte (1993) has outlined the capabilities of biomarkers as having the potential to identify the earliest events in the natural history, reducing the degree of misclassification of both disease and exposure. This opens the potential for depiction of mechanisms related to the disease pathogenesis.

There are two major types of biomarkers:

1. Biomarkers of exposure, which are used in risk prediction.
2. Biomarkers of disease, which are used in screening and diagnosis and monitoring of disease progression.

Biomarkers used in risk prediction, in screening, and in diagnostic tests are well established, and they offer distinct and obvious advantages. Molecular biomarkers have the additional potential to identify individuals susceptibility to disease (Galasko, 2001). Molecular genetics have already had an impact on neurological practice, leading to improved diagnosis. Classification of populations in terms of the degree of susceptibility on the basis of such biomarkers produces greater accuracy than relying on historical definitions of susceptibility (Gordis, 1996). For example, a biomarker will allow the stratification of a

population on the basis of a specific “genotype” associated with a disease rather than relying on a report of the “family history” of the disease. The ability to quantify “susceptibility” in this way can be an extremely important method for estimating disease risk among various populations.

1.2.3 The validity of biomarkers:

The evaluation of the validity of a biomarker is complex. Schulte and Perera (1993) suggested three aspects of measurement validity: 1) content validity, which shows the degree to which a biomarker reflects the biological phenomenon studied, 2) construct validity, which pertains to other relevant characteristics of the disease or trait, for example other biomarkers or disease manifestations, and 3) criterion validity, which shows the extent to which the biomarker correlates with the specific disease and is usually measured by sensitivity, specificity, and predictive power (Gordis, 1996).

1.2.4 Biomarkers in screening, diagnosis and prognosis:

Biomarkers depicting prodromal signs enable earlier diagnosis or allow for the outcome of interest to be determined at a more primitive stage of disease. Blood, urine, and cerebrospinal fluid provide the necessary biological information for the diagnosis. In these conditions, biomarkers are used as an indicator of a biological factor that represents either a sub- clinical manifestation or stage of the disorder.

Biomarkers used for screening or diagnosis also often represent surrogate manifestations of the disease. The potential uses of this class of biomarkers include: 1) identification of individuals destined to become

affected or who are in the “preclinical” stages of the illness, 2) reduction in disease heterogeneity in clinical trials or epidemiologic studies, 3) reflection of the natural history of disease encompassing the phases of induction, latency and detection, and 4) target for a clinical trial.

1.2.5 Specific examples of biomarkers:

Biomarkers are too many , but to name a few will point to some inflammatory ones such as interleukins (IL-1, IL-6, IL-8 and IL-18), tumor necrosis factor , acute phase proteins such as C-reactive protein and magnesium .

1.2.5.1 C-reactive protein (CRP):

C- reactive protein is a best characterized of the currently available inflammatory biomarkers and has emerged as a potential marker for cardiovascular risk (Ridker *et al*., 2002)

C-reactive protein (CRP) is an ancient highly conserved molecule, secreted by the liver in response to trauma, inflammation, and infection and decreases just as rapidly with the resolution of the condition, it is composed of five subunits , the measurement of CRP is widely used to monitor various inflammatory states that it released in the circulation plays a major role in human innate response (DuClos, 2000). CRP binds to damage tissue, to nuclear antigens and to certain pathogenic organisms in a calcium-dependent manner, it activates complement, binds to Fc receptors and acts as an opsonin for various pathogens. Interaction of CRP with Fc receptors lead to the generation of pro-inflammatory cytokines that enhance the inflammatory response. (DuClos, 2000).

Normally there is no CRP in blood serum., "a high or increasing amount of CRP in your blood suggests that you have an acute infection or inflammation. Although a result above 1mg/dl is usually considered high for CRP, most infections and inflammations result in CRP levels above 10 mg/dl.

Although the presence of C-reactive protein was found not to be specific, its disappearance from blood serum is correlated with treatment, so effect of some new drugs can now be studied in certain diseases, including malaria, with the C-reactive protein test (Naylor, 2003).

Although generally considered to be an acute-phase reactant, CRP is also produced in smooth muscle cells within human coronary arteries and is expressed preferentially in diseased vessels (Jabs *et al.,* 2003). CRP may directly affect expression of adhesion molecules, impact fibrinolysis, and alter endothelial dysfunction (Szmitko *et al.,* 2003). Very recently, transgenic mice expressing human CRP have been shown to have an increased thrombotic risk and perhaps increased atherogenesis (Danenberg *et al.,* 2003).

CRP is the most widely used of the acute phase inflammatory proteins because of its early rise and rapid kinetics (Imrie *et al*., 2007). Its half-life is 5 to 6 hours. CRP is concentrated in tissues involved in the inflammation where it exerts its biological properties. In malaria; CRP is believed to prevent entry of the sporozoite into the hepatocyte(Bachou *et al*., 2006).

Clinically, CRP can be measured with several standardized, validated, and inexpensive high-sensitivity assays (Imrie *et al*., 2007). More than 20 prospective, epidemiologic studies demonstrate that human CRP is an independent predictor of risk of myocardial infarction (MI), stroke, peripheral arterial disease, and sudden cardiac death, even in apparently healthy individuals. CRP is surprisingly specific for the prediction of vascular events (Torres and Ridker, 2003).

The rise in CRP in malaria has already been reported by some authors, CRP has also been used as a good positive predictive indicator for the diagnosis of malaria in febrile people returning from a tropical area and is a marker for malaria in epidemiological studies (Torres and Ridker, 2003).

1.2.5.2 Tumor necrosis factor (TNF):

Tumor necrosis factor alpha (TNF – alpha) is a potent cytokine with a myriad of innate immune anti-tumor properties. It is involved in systemic inflammation and it is a member of a group of cytokines that all stimulate the acute phase reaction (Imrie *et al*., 2007).

TNF primary role is in the regulation of immune cells (Kwiatkw Ski, 2005).

It causes apoptotic cell death, cellular proliferation, differentiation, inflammation, and viral replication.

Dysregulation and, in particular, overproduction of TNF have been implicated in a variety of human diseases other than cancer (Rudin *et al*., 1997).

TNF-alpha has a critical role in the bone and cartilage damage associated with rheumatoid arthritis (RA). TNF-alpha may be involved in the pathogenesis and/or progression of gestational diabetes mellitus (GDM). TNF-alpha is expressed in the myocardium during compensated pressure-overload hypertrophy and contributes to post ischemic myocardial dysfunction. The serum levels of TNF-alpha were also significantly elevated in active WG (Wagener's granulomatosis), in the stages of HIV associated disease, and in the spinal cord arthritic patients (Ubalee *et al*., 2001).

TNF is synthesized by macrophages and activated T- helper cells which in turn is responsible for the characteristics of malaria fever and induction of acute phase proteins such as (CRP) (Ubalee *et al*., 2001).

TNF synthesis in falciparum malaria is down regulated by IL2 produced by Th 2 cells and the activation of memory Th 2 cell producing IL-10 may partly be the reason for diminished malaria symptoms exhibited by many patients living in endemic areas. Despite its central role in malaria pathology , the function of TNF in controlling parasite growth should be considered. Various studies using antibodies, recombinant cytokines or knock-out mice have shown that the secretion of TNF is an important effecter of cerebral malaria (Rudin *et al*., 1997).

TNF has been shown to inhibit a mouse malaria parasite in vivo, and *Plasmodium falciparum* in vitro TNF enhances neutrophil and macrophage phagocytosis killing the sexual blood stages. Fever produced as a result of TNF release may help to limit parasite growth (Kwiatkw Ski, 2005).

1.2.5.3 Serum magnesium:

Magnesium (Mg) is present in the plant pigment cholorophyll, making plant foods a source of this mineral. We absorb about 30% to 40% of the magnesium in our diets, but absorption efficiency can increase to 80% when intakes of magnesium are low. Vitamin D, may enhance magnesium absorption (Rudin *et al*., 1997).

The diagnostic implications of changes in serum magnesium levels have become evident in recent years. Serum magnesium levels, however, like serum calcium, normally are maintained within very narrow limits so that highly accurate and specific analytical methods are required to detect the relatively small changes which may occur in certain disease states (Naylor, 2003).

The key pathogenic event in acute falciparum malaria infection is the hemolysis of both infected and uninfected red blood cells. Therefore, the increased serum magnesium concentration might occur because of the hemolysis since red blood cells contain high amounts of magnesium. Thus, the increased serum magnesium has potential application as a biomarker of acute falciparum malaria infection in adults (Imrie *et al*., 2007).

CHAPTER TWO
MATERIAL AND METHODS

2.1 Material:

2.1.1 Study population:

A total of thirty two school children whose ages ranged between five to sixteen years old were chosen at random from Senar area. Sixteen of these children were with a medical history of malaria. The remaining sixteen were apparently healthy, and they were selected to serve as a control group.

Consent of children involved was taken from their teachers and parents.

2.1.2 Samples

Three ml of blood sample were collected from each subject by venipuncture. Blood samples were collected in the morning (8-10 a.m.). Samples were allowed to clot and serum was immediately separated by centrifugation for 10 min. Serum was then stored at 4ºC and then analyzed.

2.1.3 Equipment

1. Photometer: Bio systems BTS – 310 Photometer.
2. Pipettes:
    - Manual pipette was used for pipetting samples and standards at the beginning of the assay.
    - Automatic pipettes delivering 200µl 500µl and 1 ml were used for subsequent reagent additions.

3. Vortex mixer: GENIE 2.
4. Test tubes with round bottomed 12 × 75 mm glass.
5. Magnetic racks and separators compatible with 12 × 75 mm test-tubes.
6. 37ºC water bath.
7. Refrigerator.
8. Timer
9. Various glassware including measuring cylinder beakers and reagent bottles of varying capacities.

2.1.4 Reagents:

2.1.4.1 Reagents for serum tumor necrosis factor (TNF) estimation:

- TNF-alpha standards
- Biotinylated TNF-alpha antibody
- Streptavidin-peroxidase conjugate (SPconjugate)
- Mixed diluent concentrate
- Wash buffer concentrate
- Chromogen substrate
- Stop solution.

2.1.4.2 Reagents for serum c-reactive protein (CRP) estimation:

- C-reactive protein antibody
- Immage immunochemistry systems wash solution
- Buffer
- Diluent

2.1.4.3 Reagents for serum magnesium estimation:

- Methylthymol blue (MTB)
- Barium salt of (oxyethylenenitrilo)
- Tetra acetic acid
- (Ba.EGTA),
- Buffer.

2.2 Methods

Estimation of (TNF) is carried out by enzyme linked immuno-sorbent assay (Elisa) described by (Ho, *et al*.,1986) Quantitative determination of human C-reactive protein in serum is done by rate nephelomertry and serum magnesium is measured by a modification of Methylthymol blue complexometric methods.

2.2.1 Tumour necrosis factor (TNF) estimation:

The enzyme linked immunosorbent assay (Elisa technique) is used for the measurement of (TNF) in human serum. The time required to complete an assay is approximately 5 hours.

Principle:

A murine monoclonal antibody specific for human TNF-alpha has been recoated onto a microplate. TNF-alpha in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for human TNF.

Assay procedure:

- All reagents, working standards and samples were prepared. All reagents were then brought to room temperature before use. The assay was performed at room temperature (20-30ºC).
- Excess microplate strips were removed from the plate frame and returned immediately to the foil pouch with desiccant inside. The pouch was resealed securely to minimize exposure to water vapour and stored in vacuum desiccators.
- 50 µl of standard or sample were added to each well and then microplates were covered and incubated for two hours. The timer was started after the last sample addition.
- Washing was done five times with 200 µl of Wash Buffer. The plate were inverted to decant the contents, and hit 4-5 times on absorbent paper towel to remove liquid at each step.
- 50 µl of Biotinylated TNF-alpha antibody were added to each well and incubated for two hours.
- Washing was done five times with 200 µl of wash buffer as above.
- 50 µl of chromogen substrate were added to each well and incubated for approximately 15 minutes or till the optimal blue color density developed. The plate was taped gently to ensure through mixing and the bubbles in the well were broken with a pipette tip.
- 50 µl of stop solution were added to each well . The color was changed from blue to yellow.
- The absorbance was read at a wavelength of 450 nm immediately.

2.2.2 C-reactive protein (CRP) estimation:

Principle:

CRP levels was measured by nephelomerty method described by Bland and Altman (1986) .

Procedure:

After setup, reagents, coded calibrators, controls and samples were loaded onto the system and then analyzed.

2.2.3 Serum magnesium estimation:

Principle:

Methylthymol blue MTB forms a blue complex with magnesium. Calcium interference is minimized by forming a complex between calcium and Ba-EGTA (chelating agent). The amount of MG-MTB complex formed is proportional to the magnesium concentration and is measured using a dichromatic (600 and 510 nm) endpoint technique described by Mann and Yoe 1956 .

Procedure:

This was performed on the dimension clinical chemistry system after it had been calibrated.

Test steps:

Sampling, reagent delivery, mixing, processing, and printing of results were automatically performed by the dimension system, based on the procedure of spectrophotometery .

2.2.4 Statistical analysis:

These were carried out using samples -t test, Standarad error and correlations.

CHAPTER THREE

RESULTS

3.1 Serum biomarkers:

3.1.1 Serum c-reactive protein (CRP) levels:

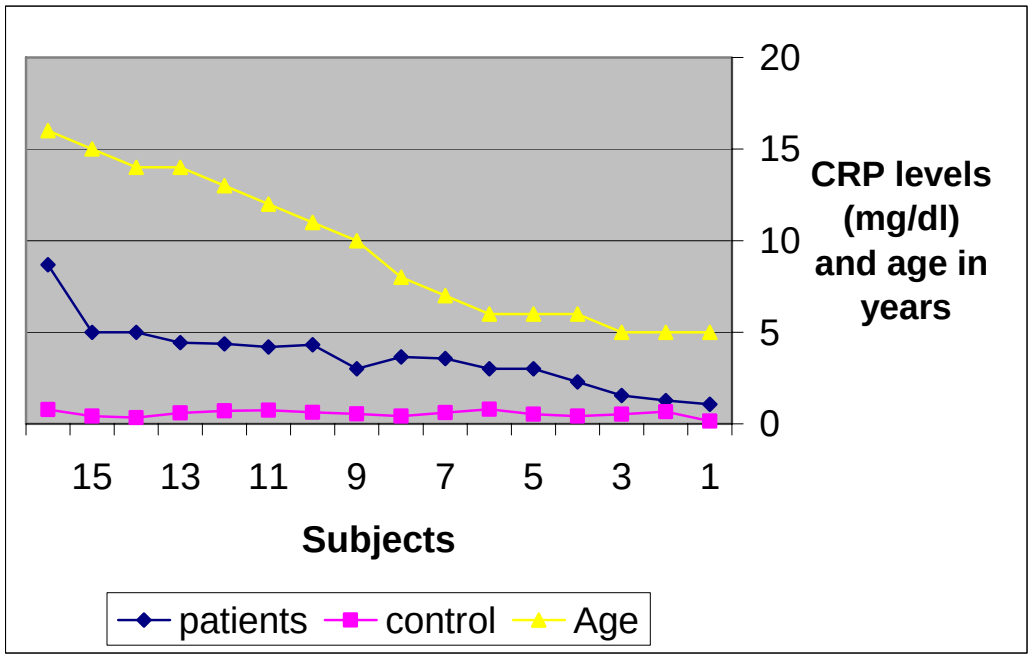
The levels of C-reactive protein (CRP) in Malaria patients and control group, expressed as means ± standard error (S.E) are shown in table (1), and presented in figure (5). CRP levels of malaria patients at 3.55 ± 1.52 are found to be significantly higher ($P < 0.003$). compared to the control group. Referance value of serum CRP is 1– 1.5 mg/dl .

Table (1): C-reactive protein (CRP) in malaria patients and the control group.

Group	CRP (mg/dl) (mean ± SE)
Control (n=16)	3.55 ± 1.52
Patients (n=16)	1.55 ± 0.58

n= number of specimens.

Figure (5): The C-reactive protein profiles in malaria patients and the control group.



❖ Every patient had a control subject having the same age .

3.1.2 Serum magnesium levels:

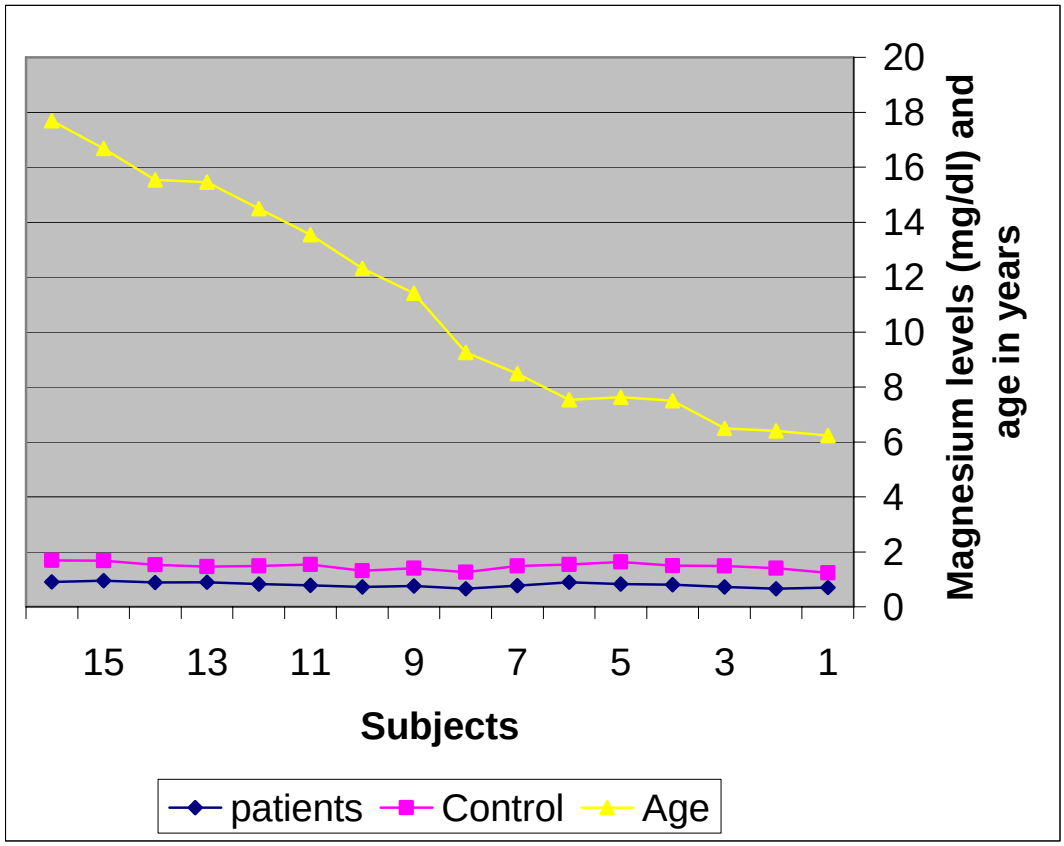
The serum levels of magnesium in the patients and the control group were shown in table (2) and profiles illustrated in figure (6). The mean value of serum magnesium in patients was compared with mean value of the control group, which was found to be significantly lower (P value < 0.004) . Referance value of serum magnesium is (1.8 – 3 mg/dl)

Table (2): Serum magnesium in malaria patients and the control group.

Group	Serum (mg/dl) (mean ± SE)
Control (n=16)	1.11 ± 0.68
Patients (n=16)	1.85 ± 0.79

n= number of specimens

Figure (6): Serum magnesium profiles (mg/dl) in malaria patients and the control Group:



- Every patient had a control subject having the same age .

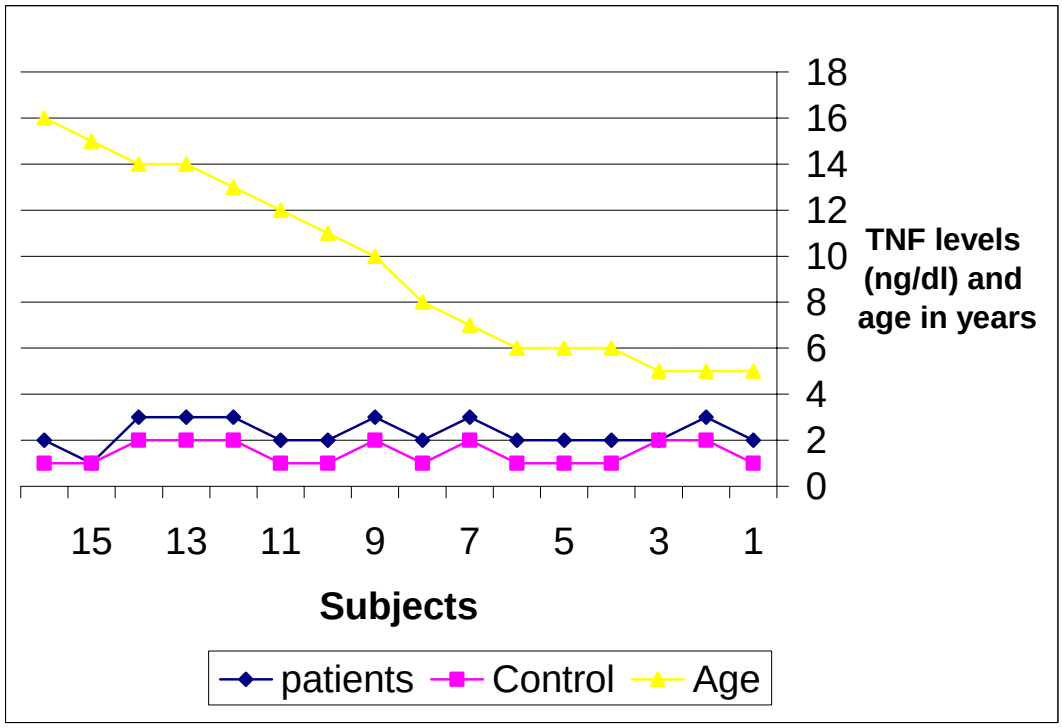
3.1.3 Tumor necrosis factor (TNF – alpha) levels:

Table (3) shows the serum levels of tumor necrosis factor – alpha, which is illustrated in figure (7) . The mean levels of TNF – alpha in patients are higher than the upper normal range.(referance value of serum TNF – alpha is 1– 2 ng/dl). However , the control group level is within the normal reference value. TNF mean levels in patients and the control group indicated that the TNF is signficantly higher among infectd children ($P < 0.002$).

Table (3): Tumor necrosis factor (TNF – alpha) levels in malaria patients and the control group.

Group	TNF level (ng/dl) (mean ± SE)
Control(n=16)	2.69 ± 0.74
Patients(n=16)	1.50 ± 0.18

Figure (7): TNF profiles in malaria patients and the control Group in (ng/dl):



❖ Every patient had a control subject having the same age .

3.1.4 Hemoglobin levels:

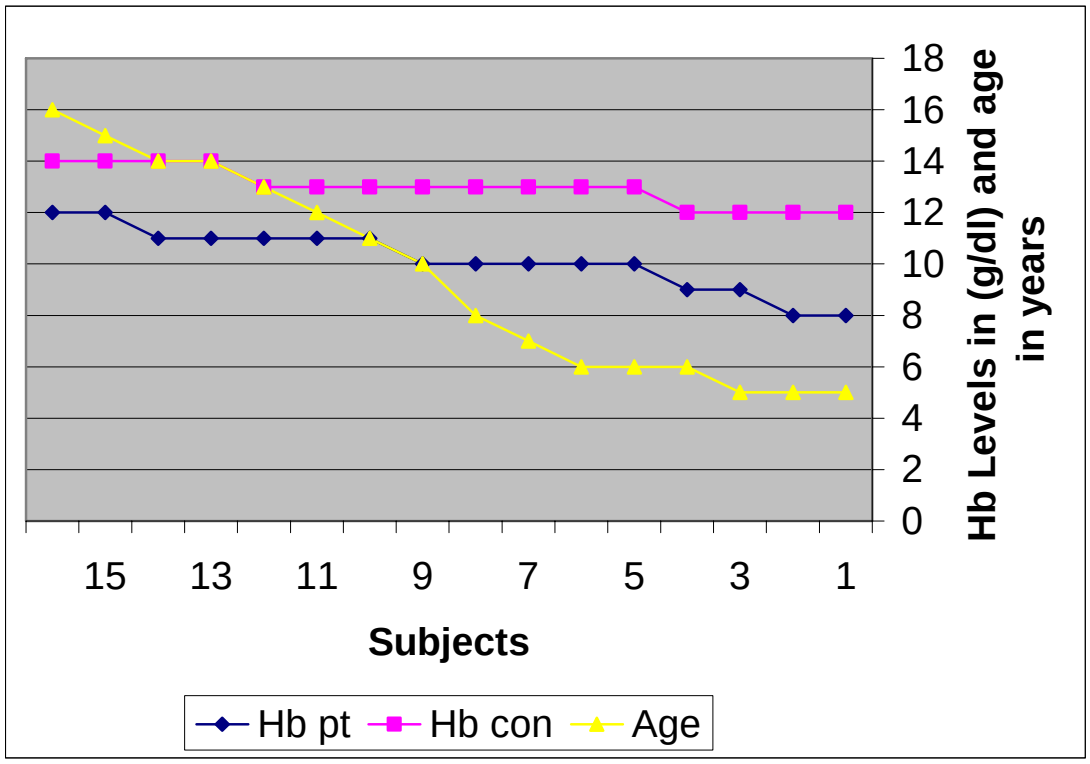
Levels of hemoglobin in patients and the control group are shown in table (4) and in figure (8). The levels of hemoglobin in patients were found to be significantly (P value < 0.001) lower than the control group , (referance value of hemoglobin is 12 – 14 g/dl in females , 14- 16 g/dl in males).

Table (4): Hemoglobin levels in malaria patients and the control group.

Group	hemoglobin (g/dl) (mean ± SE)
Control (n=16)	10.9 ± 1.22
Patients (n=16)	13.0 ± 2.21

n= number of specimens

Figure (8): Hemoglobin profiles in patients and the control group in (g/dl)



❖ Every patient had a control subject having the same age .

3.2 Correlations between biomarkers and other factors among the patients group:

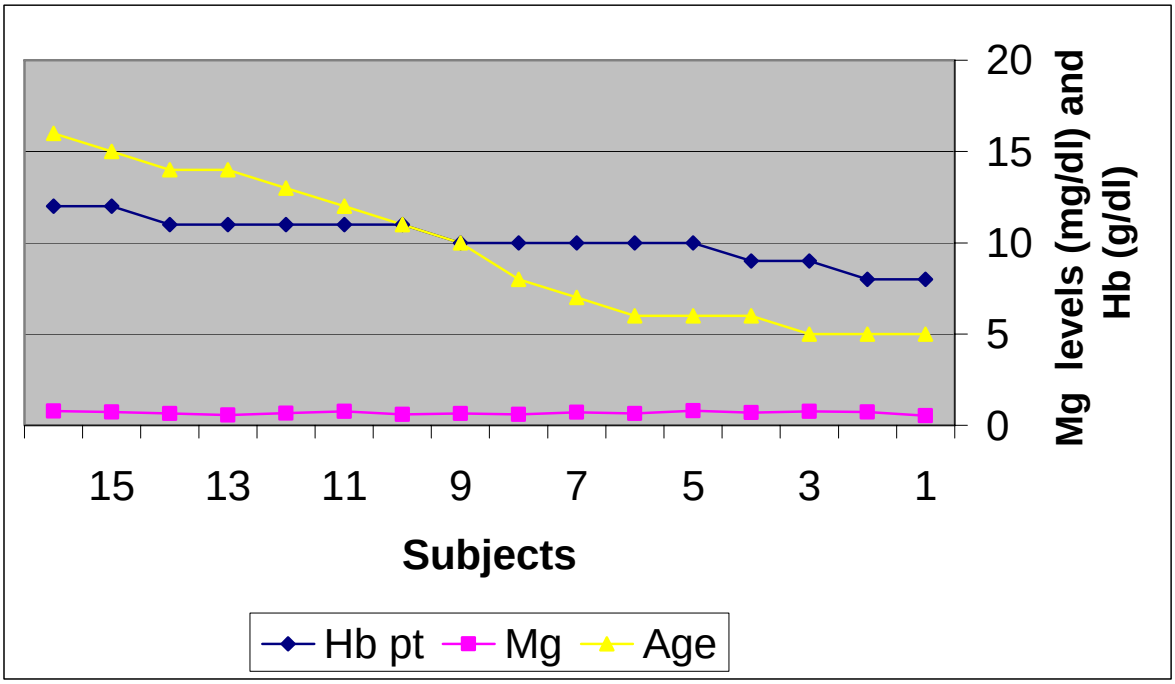
3.2.1: Correlation between serum magnesium and hemoglobin (Hb):

Table (5) shows the relation between serum magnesium and hemoglobin levels in the patients group illustrated in figure (9). Serum magnesium was found to be low and the hemoglobin levels were low in malaria patients , There was a negative correlation between the two groups .

Table (5): Correlations between serum magnesium (mg/dl) and hemoglobin levels in (g/dl) among patients.

Parameter	correlation	(mean ± SE)
Serum magnesium Hb	0.06	1.11 ± 0.68 10.9 ± 1.22

Figure (9): Correlation, between Serum magnesium and hemoglobin (Hb) in malaria patients.



❖ Every patient had a control subject having the same age .

CHAPTER FOUR

DISCUSSION

4.1 Serum inflammatory biomarkers during malaria infections:

4.1.1 Serum C-reactive protein:

Human C-reactive protein (CRP) is clinically important in classical acute phase response (APR) (Imrie *et al*., 2007).

Many analysis showed that CRP levels were significantly associated with splenomegaly, fever, hemoglobin, and age. CRP levels also increased with increasing parasitemia in malaria infections (Imrie *et al*., 2007).The present study is in agreement with previous similar reports. This inflammatory biomarker though definitively high in malaria infection can not be used as a diagnostic parameter. However , it may be of value in prognosis and other aspects of this biomarker such as timing of production , intensity of effects during various stages of the disease which may prove to be important in terms of treatment .

4.1.2 Serum magnesium level:

Serum magnesium concentration was measured in another study (Anderson *et al*., 2000) in 4 adult patient (age range: 18–40 yr) presenting with uncomplicated *falciparum* malaria infection and a control

group. Magnesium concentration in the patients was 1950.0 ± 10.0 μg/dl. The control serum magnesium was 640.0 ± 40.0 μg /dl. This represents an over threefold increase in serum magnesium levels above normal value ($P<0.01$). The key pathogenic event of acute *falciparum* malaria infection is the hemolysis of both infected and uninfected red blood cells. Therefore, the increase of serum magnesium concentration might occur because of the hemolysis as red blood cells contain high amounts of magnesium. The increased serum magnesium has potential application as a biomarker of acute *falciparum* malaria infection in adults (Anderson, *et al*., 2000).

The present study showed that the serum Mg levels in malarial patients was lower compared with their counterparts the control group which was below normal reference value , this raises some questions pertaining to whether Sudanese at large have different reference range. reference value have been recently questioned regarding ranges. A suitable example for this point , there is an ongoing debate on narrowing the TSH reference range in adults ,within the range of normal for thyroid hormone concentration, there is a rather wide degree of variation from individual to individual (Arem,2000) .

The following reference ranges represent commonly used thyroid function reference ranges. However, ranges and units of measurement may vary from one laboratory to another. Patient results must be compared to the reference range of the appropriate testing facility (Arem, 2000) .

Adult Reference Ranges:

T4 = 5.6-13.7 ug/dl (mcg/dl)

FT4 = 0.8-1.5 ng/dl

T3= 87-180 ng/dl

FT3 = 230-420 pg/d;

TSH = 0.4-4.5 mIU/L (mU/L)

4.1.3 Serum tumour necrosis factor:

Tumour necrosis factor alpha (TNFα) is thought to play a role in the development of immunity in malaria infectious.
Some malaria disease severity is attributed to the induction of the pro-inflammatory cytokines TNF-alpha (Sinha *et al*., 2008) .

Susceptibility/resistance to plasmodium falciparum malaria has been correlated with polymorphisms in more than 30 human genes with most association analyses having been carried out on patients from Africa and Southeast Asia. Significantly higher TNF levels were observed in patients with severe malaria (Sinha *et al*., 2008).

Plasma concentration of TNF-alpha and IFN-gamma were higher in parasitaemic than aparasitaemic individuals and donors who had clinical malaria had higher levels of TNF-alpha.There was appositive correlation between age of the individual and the concentration of plasma TNF-alpha and IFN-gamma suggesting that the production of these cytokines could be modulated by repeated malarial infections (Carter *et al.*, 2005).

The findings in the present study is in agreement with the findings of other workers, where peripheral levels of TNF, and ferritin were found to be elevated during Placental malaria. (Kabyemela *et al*., 2008).

High level of serum TNFα correlates with resistance to malaria infection which agrees with the findings of the present study that TNFα level was significantly elevated in the infected children compared to their counterpart control group, Whether this is an indication of different mechanisms of pathogenesis or reflects differences in immunological responses at different stages of human life remains to be investigated.

4.1.4 Hemoglobin levels:

Anemia or low hemoglobin commonly occurs in chronic infection, inflammation and malignancy. In the present study hemoglobin levels in malaria patients were significantly low (10.9 ± 1.22) and some of them were suffering from anemia compared to their counterpart control group. When these children encounter the malaria their hemoglobin was decreased but not to extent of affecting serum magnesium levels. In this situation one is question about the sensitivity and specificity of quantifying method used for estimation, or perhaps if the mineral chelated during the process.

This study showed that there is no direct correlation between hemoglobin and serum magnesium levels among malaria patients which may be attributed to other factors affecting the magnesium levels in these children .

CHATER FIVE
CONCLUSION AND RECOMMENDATION

5.1 Conclusion

It's concluded from the present investigations that the malaria can affect some inflammatory biomarkers such as c-reactive protein concentrations which were increased in malaria patient and of high incidence among children. In accordance with other studies there is a clear difference in serum magnesium concentration in the two groups (patients and control) that the malaria patients had levels below the normal range. TNF level is increased among patients, hemoglobin was low among these children, also there is no clear relationship between these factors such as age, and hemoglobin and c-reactive protein and serum magnesium respectively.

5.2 Recommendations

* More investigations are needed to resolve the complexity of the issue.

* Attention should be given to the malaria problems in the country in a coordinated manner.

* Socioeconomic, environmental and genetical factors as underlying causes of inflammatory biomarker imbalance during malaria infections. Which should be addressed and investigated through maltidisciplinary teams.

* Parallel study need to be done in adults (divided into males and females).

* Cohort studies should be encouraged and facilitated though governmental bodies and the private sector, which will benefit future work and planning regarding infectious disease and inflammatory biomarkers.

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